Phorbol Myristate Acetate-Mediated Stimulation of Transcytosis and Apical Recycling in MDCK Cells

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Abstract. We observed that phorbol myristate acetate (PMA) stimulates transcytosis of the polymeric immunoglobulin receptor (pIgR) in MDCK cells. Apical release of pre-endocytosed ligand (dimeric IgA) bound to the pIgR can be stimulated twofold within 7 min of addition of PMA while recycling of the ligand from the basal surface is not affected. In addition, apical surface delivery of pIgR and cleavage of its ectodomain to secretory component (SC) is also stimulated by PMA. The recycling of apically internalized ligand back to the apical surface is similarly stimulated. These results suggest that the stimulation of apical delivery is from an apical recycling compartment. The effect of PMA suggests that protein kinase C (PKC) is involved in the regulation of pIgR trafficking in MDCK cells. To test this we down regulated PKC activity by pre-treating cells with PMA for 16 h and observed that transcytosis could no longer be stimulated by PMA. Western blots show that the PKC isozymes α and to a lesser extent ϵ, are depleted from MDCK cells which have been pre-treated with PMA for 16 h and that treatment of MDCK cells with PMA for 5 min causes a dramatic translocation of the PKC α isozyme and a partial translocation of the ϵ isozyme from the cytosol to the membrane fraction of cell homogenates. This translocation suggests that the α and/or ϵ isozymes may be involved in PMA mediated stimulation of transcytosis. A mutant pIgR in which serines 664 and 726, the major sites of phosphorylation, are replaced by alanine is stimulated to transcytose by PMA, suggesting that phosphorylation of pIgR at these sites is not required for the effect of PMA. These results suggest that PMA-mediated stimulation of pIgR transcytosis may involve the activation of PKC α and/or ϵ, and that this stimulation occurs independently of the major phosphorylation sites on the pIgR. Finally, PMA stimulates transcytosis of basolaterally internalized transferrin, suggesting that PMA acts to generally stimulate delivery of endocytosed proteins to the apical surface.

The plasma membrane of polarized epithelial cells contains two surfaces: an apical surface facing the external world or lumen of a cavity, and a basolateral surface facing adjacent cells and underlying connective tissue. These two plasma membrane surfaces have very different protein compositions. To establish and maintain these different compositions, plasma membrane proteins must be continually sorted (reviewed in Hopkins, 1991; Mostov et al., 1992; Rodriquez-Boulan and Powell, 1992). There are two routes by which proteins reach the correct membrane surface. First, newly made plasma membrane proteins are synthesized in the RER and sent through the Golgi to the TGN, where they can be sorted into vesicles that deliver them to the correct surface. Alternatively proteins can be delivered to one membrane surface, generally basolateral, and then endocytosed and delivered to endosomes. Here proteins are sorted for either recycling to the basolateral surface or transcytosis to the opposite surface. Transcytosis to the apical surface is the only route for apical delivery of plasma membrane proteins that has been demonstrated in all epithelial cells so far examined. In some cells, such as hepatocytes, transcytosis is virtually the only route by which plasma membrane proteins reach the apical surface.

One of the best understood model systems for studying transcytosis is the polymeric immunoglobulin receptor (pIgR)[1] (reviewed in Aroeti et al., 1992). This integral membrane protein is targeted from the TGN to the basolateral surface, where it can bind its ligand, dimeric IgA (dIgA). The receptor can then be endocytosed and transcytosed to the apical surface. Before reaching the apical surface, a sig-

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1. *Abbreviations used in this paper*: ARF, ADP-ribosylation factor; BFA, brefeldin A; DAG, 1,2 diacylglycerol; dIgA, dimeric IgA; pIgR, polymeric immunoglobulin receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13 acetate; SC, secretory component; Tf, transferrin; TfR, transferrin receptor; WT, wild type.

nificant fraction of the transcytosing IgA is delivered to a tubulo-vesicular compartment located subjacent to the apical plasma membrane (Hunziker et al., 1990; Barroso and Sztul, 1994; Apodaca et al., 1994). This compartment is also accessible to membrane-bound markers that are endocytosed from, and recycle to, the apical surface, and will therefore be referred to here as the apical recycling compartment. We have recently found that transferrin, which is normally endocytosed from, and recycles to the basolateral surface, can partially reach this apical recycling compartment (Apodoca et al., 1994). When IgA reaches the apical membrane the extracellular, ligand-binding portion of the pIgR is cleaved off and released into medium overlying the apical surface. This cleaved fragment is called secretory component (SC). The pIgR has been expressed from cloned cDNA in MDCK cells. When grown on permeable filters, these cells form a well-polarized epithelial monolayer and the exogenous pIgR functions as in vivo (Mostov et al., 1986).

Transcytosis of the pIgR is regulated by at least two signals, phosphorylation of Ser^{664} and binding of dIgA. A major site of phosphorylation of the pIgR is at Ser^{664}, located in the COOH-terminal cytoplasmic domain of the pIgR (Casanova et al., 1990). If this residue is mutated to a nonphosphorylatable Ala, (pIgR Ala^{664}) transcytosis of the pIgR that does not have dIgA bound ("empty pIgR") is greatly reduced. Conversely, mutation of this Ser to an Asp, whose negative charge can mimic a phosphate, enhances transcytosis of the empty pIgR. Binding of dIgA also stimulates transcytosis (Hirt et al., 1993; Song et al., 1994). While dIgA binding to the wild-type pIgR (pIgR WT) stimulates transcytosis about 30-40%, dIgA binding to pIgR Ala^{664} increases transcytosis more than 200%. The signals of Ser^{664} phosphorylation and dIgA binding are therefore relatively independent, in that either one can stimulate transcytosis in the absence of the other. However, maximal transcytosis is observed only when both signals are present.

In addition to Ser^{664}, a second major site of phosphorylation of the pIgR is Ser^{726} (Hirt et al., 1993). Ser^{726} is in a consensus sequence for phosphorylation by several major classes of kinases, including protein kinase C (PKC) cAMP-dependent kinase, and casein kinase II (Kennelly and Krebs, 1991). The importance of this potential site for PKC phosphorylation in pIgR traffic led us to examine the role of PKC in regulating the movement of the pIgR. The role of PKC in various biological processes has been frequently investigated by using phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) (Nishizuka, 1992). Ordinarily PKC is activated by 1,2-diacylglycerol (DAG) that is produced by activation of phospholipase C. This activation is generally short-lived. PMA can substitute for DAG, although the effects of PMA can be very long lasting. Treatment of cells with PMA has a wide range of effects on the movement of various cell surface receptors that are involved in receptor mediated endocytosis (reviewed in Backer and King, 1991). For instance, depending on the receptor and the cell type, PMA can increase or decrease the number of receptors on the cell surface. These changes can be the result of alterations in internalization or externalization of receptors, or both.

PKC is a family of isozymes (Nishizuka, 1992). The classic members of the family include isozymes α, βI, βII, and γ, which are regulated by DAG, phosphatidylserine, and Ca^{2+}. The other known PKC isozymes, δ, ϵ, ζ, η, and θ, lack the Ca^{2+} binding C2 domain and are therefore Ca^{2+} independent. When not activated, PKC is generally found in the cytosol. Upon activation the PKC molecules are translocated to the particulate fraction of the cell. This translocation appears to involve binding of PKC to intracellular receptors, termed RACKs (Mochly-Rosen et al., 1991).

In this study we have treated MDCK cells with PMA to investigate whether PKC is involved in the regulation of pIgR traffic in MDCK cells. We found that PMA caused a rapid increase in the rate of pIgR transcytosis and appeared to stimulate movement of pIgR from the apical recycling compartment to the apical surface. This stimulation was apparently due to the involvement of PKC, (probably the α or ϵ isozyme). Phosphorylation of Ser^{726} does not, however appear to be required for this stimulation. We also found that PMA stimulated recycling of apically endocytosed pIgR to the apical surface, and stimulated transcytosis of transferrin from the basolateral to the apical surface. These results suggest that PMA may generally stimulate delivery of membrane-bound material from the apical recycling compartment to the apical surface.

Materials and Methods

Materials

All chemicals were reagent grade. PMA and 4α-phorbol (Sigma Immunochemicals, St. Louis, MO) were dissolved in acetone at a concentration of 1 mg/ml and stored at −20°C. Human dIgA was kindly provided by Dr. J. P. Vaerman (Catholic Univ. of Louvain, Brussels, Belgium). Iodination of dIgA was performed according to the iodine monochloride protocol described previously (Breitfeld et al., 1989*b*). Iodinated dIgA preparations averaged 1.0×10^7 cpm/μg. Protein A-Sepharose was purchased from Pharmacia. [^{35}S]Cys and [^{32}P]orthophosphate were from Du-Pont-New England Nuclear (Boston, MA). PKC isozyme-specific antibodies were purchased from Seikagaku America (Rockville, MD) and GIBCO BRL (Gaithersburg, MD). Minimal essential medium was purchased from Mediatech (Washington, DC). Cys- and Met-free DME was purchased from ICN Radiochemicals (Irvine, CA). The penicillin, streptomycin, and fungizone cocktail was obtained from the Cell Culture Facility at the University of California (San Francisco, CA). FBS was purchased from Hyclone (Logan, UT).

Transcytosis Assays

Postendocytotic fate assays for dIgA were performed as described previously (Breitfeld et al., 1990). MDCK cells expressing the wild type or mutant rabbit pIgR were cultured on 12-mm Costar Transwell cell culture chamber inserts, 0.4-μm pore size (Costar Corp., Cambridge, MA) and were maintained in MEM supplemented with 5% FBS and penicillin, streptomycin, and fungizone. Medium was replaced every other day, and the cells were used within 7 d of plating. Cells cultured on 12 mm filters were allowed to internalize iodinated ligand (10^5 cpm/ml in MEM) from the basolateral medium for 10 min at 37°C, or for 90 min at 17°C with a subsequent 90-min chase with MEM at 17°C. After internalization, the filters were washed extensively within a 5-min period and placed in a multiwell tissue culture dish at 37°C, with medium added apically and basally. Apical and basal media were sampled at various intervals for up to 2 h and the radioactive ligand counted in a Packard gamma counter. The transcytosed and recycled ligand were expressed as a percentage of total collected ligand in the apical and basolateral media and cells. Pulse-chase analysis of metabolically labeled receptors was performed as previously described (Breitfeld et al., 1989*b*).

Confocal Microscopy

MDCK cells expressing pIgR were allowed to internalize dIgA at 150 μg/ml, from the basal surface for 90 min at 17°C and then chased at 17°C for an additional 90 min in MEM. Cells were then either chased at 37°C for 10, 20, or 40 min with or without 2 μM PMA or not chased. All samples were then fixed with 4% paraformaldehyde using a pH-shift protocol (Bacallao et al., 1989). Cells were fixed 5 min at room temperature with 4% paraformaldehyde, 80 mM Pipes/KOH, pH 6.5, 5 mM EGTA, 2.0 mM $MgCl_2$, and then transferred to 4% paraformaldehyde dissolved in 100 mM NaBorate, pH 11.0, and incubated 10 min at room temperature. Cells were washed 2 × 3 min with PBS, pH 8.0, and nonreacted paraformaldehyde was quenched 10 min at room temperature with 75 mM NH_4Cl, 20 mM Glycine, pH 8.0, dissolved in PBS, pH 8.0. Cells were washed with PBS 2 × 5 min and nonspecific sites were blocked with PBS-0.7% (wt/vol) fish skin gelatin-0.025% (wt/vol) saponin (Sigma Immunochemicals). The fixed cells were incubated with FITC-derivatized goat IgG raised against human IgA (Cappel Laboratories, Cochranville, IL) for 1 h at 37°C and rinsed 3 × 5 min with PBS-saponin, 1 × 5 min with PBS, 1 × 5 min with PBS containing 0.01% Triton X-100, followed by a 5-min wash in PBS, pH 8.0, alone. Cells were postfixed in 4% paraformaldehyde dissolved in 100 mM Na-cacodylate, pH 7.4, for 30 min at room temperature, washed twice and mounted in mounting medium containing *p*-phenylene diamine (Sigma Immunochemicals).

The samples were analyzed using a krypton-argon laser coupled with a BioRad MRC600 confocal head, attached to a Nikon Optiphot II microscope. The samples were scanned for FITC using a K2 filter block. Collection parameters were as follows: zoom = 2.5, 0.5 s/scan, 5 frames/image, Kalman filter, motor step size = 0.2 μM. The data was analyzed using Comos software.

Recycling from the Apical Compartment

Filter grown MDCK cells expressing the pIgR were allowed to internalize iodinated ligand (10^5 cpm/ml in MEM) from the apical medium for 15 min at 37°C. After internalization cells were washed extensively over a 5-min period and placed in a multiwell tissue dish at 37°C, with media added apically and basally. Apical and basal media were sampled at various intervals for up to 2 h and the radioactive ligand counted as above.

Translocation of PKC Isozymes

Cells were grown on 100 mm Transwell filters for 5 d and treated with 2 μM PMA in MEM at 37°C for 16 h, or 5 min, or not treated. The filters were then placed at 4°C, the medium was removed and the cells were lysed in 0.7 ml of homogenization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 17 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml aprotinin, and 5 μg/ml leupeptin), and scraped from the filters. The homogenates were centrifuged at 100,000 *g* for 30 min and the supernatants (cytosolic fraction) were collected. The pellets were resuspended in 0.7 ml of the homogenization buffer containing 0.1% Triton X-100. Equal amounts of protein were run on 10% SDS-PAGE followed by Western blot analysis using isozyme-specific monoclonal antibodies for α,β, and γ PKC isozymes followed by rabbit anti-mouse secondary antibodies. Reacting protein was detected using iodinated protein A. PKC δ, ϵ, and ζ were detected with polyclonal antibodies and iodinated protein A (Mochly-Rosen et al., 1990). The distribution of PKC isozymes in the soluble and particulate fractions for the treated and non-treated cells were quantified by PhosphorImager analysis.

Transcytosis and Recycling of Pre-internalized Transferrin

Canine apo-transferrin (Sigma Immunochemicals) was converted to saturated iron III-transferrin (holotransferrin) as described (Podbilewicz and Mellman, 1990) and labeled with [^{125}I] to a specific activity of 4.3 × 10^6 cpm/μg using ICl (Brietfeld et al., 1989*b*). MDCK cells grown on filters were allowed to internalize 5 μg/ml [^{125}I] holo-transferrin from the basal surface for 1 h at 37°C. Both sides of the filters were then washed at 4°C with PBS to remove unbound transferrin and chased in MEM containing 10 μg/ml unlabeled holo-transferrin with or without 2 μM PMA. Apical and basal media were collected at various time intervals up to 140 min and counted. The amount of transcytosed and recycled ligand was expressed as a percentage of the total counts.

Results

PMA Stimulates Transcytosis of dIgA

We have previously developed a method to quantitatively analyze the transcytosis of preinternalized dIgA in MDCK cells expressing the pIgR (Breitfeld et al., 1989*a*). Cells were allowed to internalize ^{125}I-labeled dIgA from the basolateral surface for 10 min. The cells were then washed for 5 min to remove most (>90%) of the ligand from the cell surface. At this point there was an intracellular pool of ^{125}I-labeled dIgA. The cells were then chased for up to 120 min and the release of ^{125}I-labeled dIgA into the apical medium (transcytosis) was measured and expressed as a percent of the total ^{125}I-labeled dIgA present in all fractions at the start of this chase. We found that if 2 μM PMA was added at the beginning of the chase period the rate of transcytosis of ^{125}I-labeled dIgA was increased (Fig. 1). This stimulation was most pronounced during the early time points. The total

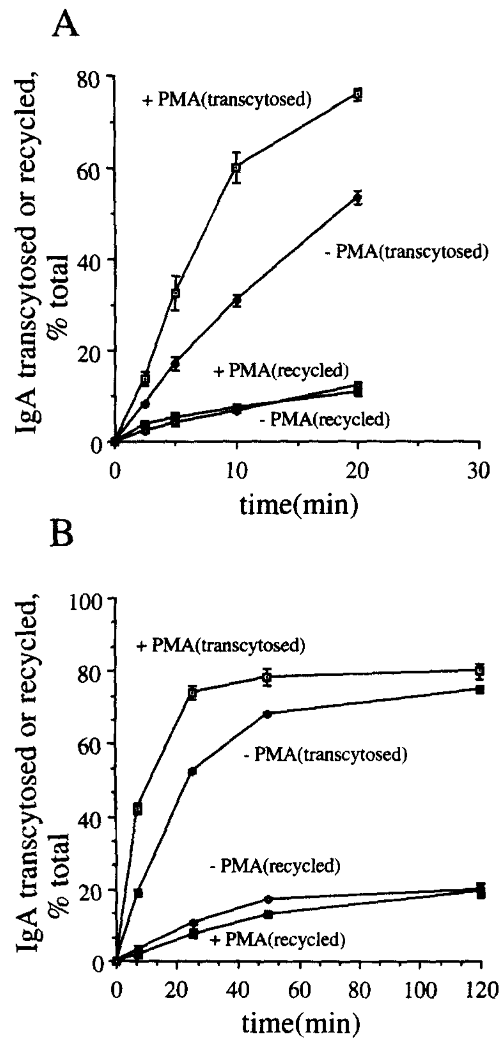

Figure 1. PMA stimulation of transcytosis of dIgA in MDCK cells expressing pIgR. Cells were allowed to internalize ^{125}I-labeled dIgA from the basolateral surface for 10 min at 37°C. The cells were then washed and chased up to 2 h at 37°C with or without 2 μM PMA in the medium. Apical and basal media were sampled at various intervals. *A* was an experiment that examined only early time points in detail. *B* was a separate experiment that focused on later time points. (It was advantageous to perform separate experiments to facilitate manipulation of the filters on a precise schedule.) Values are means ± SEM using five filters for each experiment for both PMA-treated and -non-treated cells. These results are representative of four separate experiments.

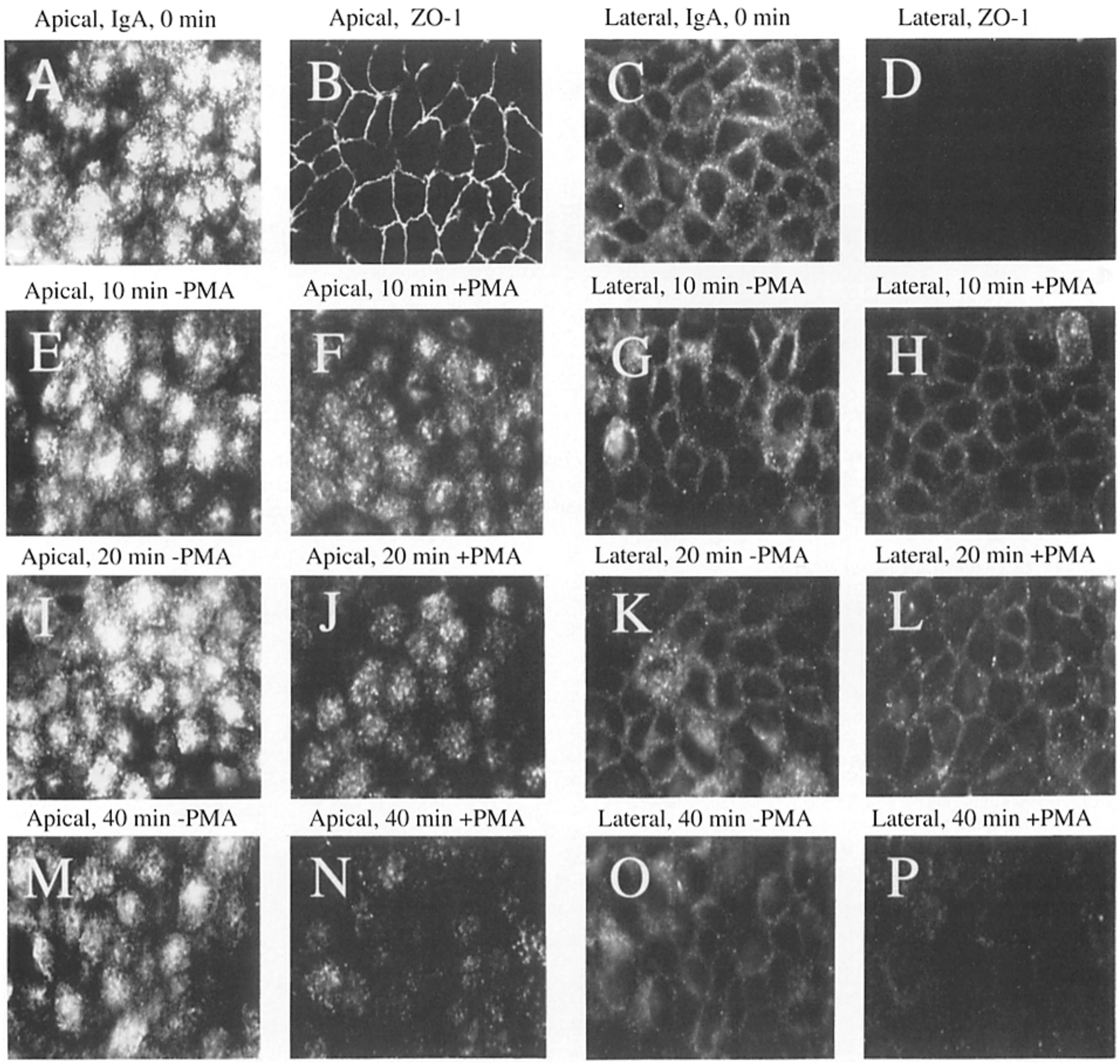

Figure 2. Morphological analysis of the effect of PMA on IgA transcytosis. IgA was internalized from the basolateral surface of MDCK cells expressing the pIgR for 90 min at 17°C and then chased for an additional 90 min at this temperature in ligand-free medium. Cells were then washed and fixed without being chased (*A–D*) or after being chased in MEM without (*E, G, I, K, M,* and *O*) or with 2 μM PMA (*F, H, J, L, N,* and *P*) for 10 min (*E–H*), 20 min (*I–L*), or 40 min (*M, N, O, P*) at 37°C. Cells were fixed with paraformaldehyde and stained for IgA in all panels except *B* and *D* which were stained with the tight junction marker ZO-1. Cells were examined with a laser scanning confocal immunofluorescent microscope. Representative individual sections are shown at the level of the apical portion of the cell at the level of the tight junctions (*A, B, E, F, I, J, M,* and *N*) or at the level of the middle of the nucleus (*C, D, G, H, K, L, O,* and *P*). All images are at the same magnification.

amount of IgA transcytosed in untreated cells approached that of the PMA-treated cells by 120 min. Neither the vehicle alone nor 4-α phorbol, which does not activate PKC, had any detectable effect on transcytosis of IgA or SC (data not shown).

In preliminary experiments we tested a wide range of concentrations of PMA and found that 2 μM gave the maximal stimulation of transcytosis. Concentrations as low as 20 nM gave a significant stimulation. The 2-μM dose proved to have an advantage over the lower doses of not perturbing the integrity of the monolayer even after long periods of exposure (see below).

We also investigated the stimulation of transcytosis morphologically using confocal microscopy. Before reaching the apical plasma membrane, transcytosing dIgA accumulates in an apical recycling compartment (Hunziker et al., 1990; Barroso and Sztul, 1994; Apodaca et al., 1994). This accumulation can be most easily seen when transcytosis is slowed by incubating cells at reduced temperature. We therefore allowed MDCK cells to internalize unlabeled dIgA for 90 min at 17°C, chased for an additional 90 min at 17°C, and then warmed to 37°C for up to 40 min in the presence or absence of 2 μM PMA. Cells were then fixed and the localization of the internal dIgA was determined by indirect immunofluorescence and laser scanning confocal microscopy. In Fig. 2 the top row (*A–D*) is from cells that were not chased;

the second row (*E–H*) are cells chased for 10 min; the third row (*I–L*) are cells chased for 20 min; and the bottom row (*M–P*) are cells chased for 40 min. In all cases the left two columns (*A, B, E, F, I, J, M,* and *N*) are from an optical section near the top of the cell, and are designed to show the accumulation of IgA in the apical recycling compartment. The right two columns (*C, D, G, H, K, L, O,* and *P*) are from an optical section near the middle of the cell at the level of the nucleus, and are designed to mainly show IgA in the cytoplasm underlying the lateral plasma membrane. This IgA is probably mostly in basolateral early endosomes. However, due to unevenness in the filter and monolayer, in occasional cells in these panels the plane of the optical section runs above the nucleus. In these few cells IgA that has accumulated in the apical cytoplasm can be seen. To confirm the orientation in the cell, in the top row (only), *B* and *D* are from a second channel that shows the staining of the tight junctions with a monoclonal antibody against ZO-1. As expected, ZO-1 staining is seen only at the top of the cell (*B*), and not in the middle region of the cell (*D*). None of the 0 time samples (i.e., *top* row) were exposed to PMA. For the 10-, 20-, and 40-min chase times, cells in the second and fourth columns (i.e., *F, H, J, L, N,* and *P*) were treated with PMA during the chase, while the untreated matched controls are in the first and third columns (*E, G, I, K, M,* and *O*).

After endocytosis under these conditions, but before any chase at 37°C, the greatest concentration of IgA is primarily in the apical recycling compartment (*A*), but substantial ligand was also found throughout the lateral cytoplasm surrounding the nucleus (*C*). Incubation of the cells at 37°C for up to 40 min without PMA caused a gradual release of IgA from both the apical recycling compartment (*E, I,* and *M*) and from the lateral region of the cytoplasm (*G, K,* and *O*). Treatment of the cells with PMA caused a rapid disappearance of IgA, due to apical release. Loss of IgA from the apical recycling compartment could be easily detected after only 10 min (compare *F* to the control *E*), although further loss was seen at later times (compare *J* to *I*, and *N* to *M*). Some PMA-stimulated release of IgA from the lateral cytoplasm did occur; this was more evident at the longer times (compare *H* to control *G*, *L* to *K*, and *P* to *O*). Similar results have been obtained when the dIgA was initially internalized for 10 min at 37°C (not shown).

To permit a direct comparison of these morphological data with a quantitative, biochemical analysis, we compared the effect of PMA on the rate of transcytosis when cells were allowed to endocytosed ^{125}I-labeled IgA under the same conditions as the confocal experiment. Fig. 3 represents the result when ^{125}I-labeled IgA is endocytosed at 17°C for 90 min and cells were chased for another 90 min at 17°C before being chased at 37°C with or without PMA. This data shows that the PMA-mediated increase in the rate of IgA released is similar in cells loaded with IgA under either of the two loading conditions.

PMA Stimulates Recycling of Apically Internalized IgA

We have recently found that apically endocytosed membrane markers can reach the same apical recycling compartment as the transcytosing dIgA (Apodaca, G., and K. Mostov, unpublished). A convenient apically endocytosed marker is dIgA. When the pIgR is transcytosed to the apical surface, its cleavage to SC is not very rapid and consequently there is a pool of uncleaved pIgR that can be endocytosed from the apical surface. Dimeric IgA added to the apical medium can therefore bind to this apically exposed pIgR and then is internalized and delivered to the apical recycling compartment (Breitfeld et al., 1989*a*). This apically internalized dIgA ordinarily is almost entirely recycled to the apical surface. We investigated whether PMA could stimulate the recycling of apically internalized dIgA back to the apical surface. We added ^{125}I-labeled dIgA to the apical medium for 15 min at 37°C to allow binding and endocytosis, washed away the ligand and then chased for up to 120 min in ligand-free medium. Fig. 4 shows that the rate of apical recycling was stimulated by PMA while basal release was not stimulated. This result is consistent with our hypothesis that PMA stimulates delivery from this apical compartment to the apical surface.

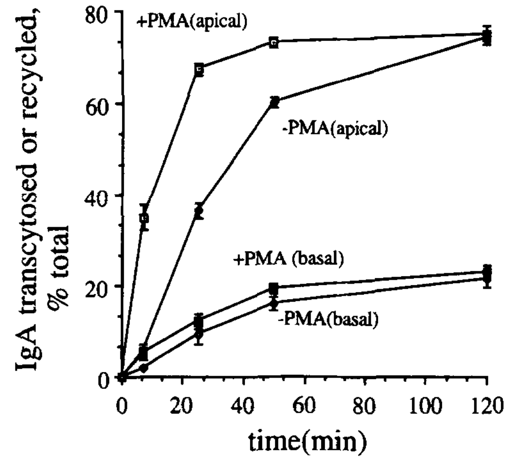

Figure 3. PMA-mediated stimulation of IgA transcytosis in cells allowed to endocytose at 17°C for 90 min. IgA was internalized from the basolateral surface of MDCK cells expressing the pIgR for 90 min at 17°C and then incubated for an additional 90 min at this temperature in ligand-free medium. Cells were then washed and chased for up to 2 h at 37°C in MEM without or with 2 μM PMA, and the apical and basolateral chase media were collected. At the end of the 37°C chase, radioactivity associated with all fractions, including the filters, was determined. Values are means of four filters ± SEM. These results are representative of three separate experiments.

Stimulation of Transcytosis Is Not Due to Increased Leakiness of the Bilayer

It has been reported that treatment of MDCK cells with 10 nM PMA increases the permeability of MDCK cell monolayers (Ojakian, 1981). We were concerned that the effects of PMA that we observed on transcytosis might be due to leakage of dIgA between cells. This did not seem very likely, because the experiment in Fig. 1 measured the transcytosis of pre-internalized dIgA, not dIgA continuously present in the basolateral medium. Also, in Fig. 1 basolateral release of ^{125}I-labeled dIgA was not stimulated, which might be expected if leakage occurred. Nevertheless, we measured the integrity of the tight junctions by examining the ability of [^{14}C]inulin to diffuse across monolayers that had been treated with various concentrations of PMA for either 20 or 210 min. In agreement with previous reports we found that 20 or

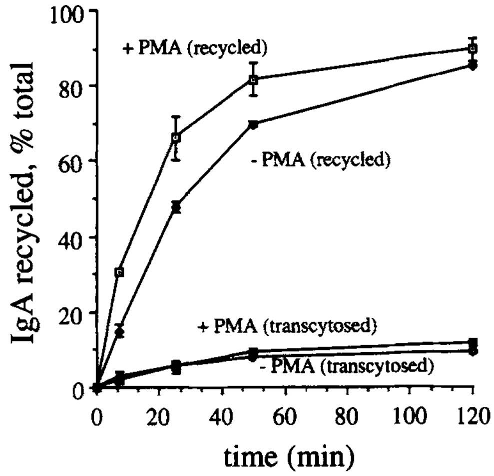

Figure 4. Effect of PMA on recycling of dIgA from the apical compartment. ^{125}I-labeled dIgA was endocytosed from the apical surface for 15 min at 37°C. Cells were washed and chased for up to 120 min with or without 2 μM PMA added to the media. Samples were taken from the apical and basal media and counted. Values are expressed as a percent of the total radioactive material initially endocytosed and are a mean ± SEM, N = 6.

200 nM PMA increased the permeability of the monolayer at 210 min (Fig. 5). With 2 μM PMA, however the permeability of the monolayer was slightly less than the non-treated control monolayer. We do not know the reason for this biphasic response. We also measured the trans-monolayer electrical resistance of the monolayers treated for up to 16 h with 2 μM PMA and found no effect (not shown). These data suggest that the stimulation of transcytosis by PMA is not due to increased leakiness of the monolayer.

PMA Stimulates Release of SC into the Apical Medium

We also investigated the effect of PMA on the transcytosis

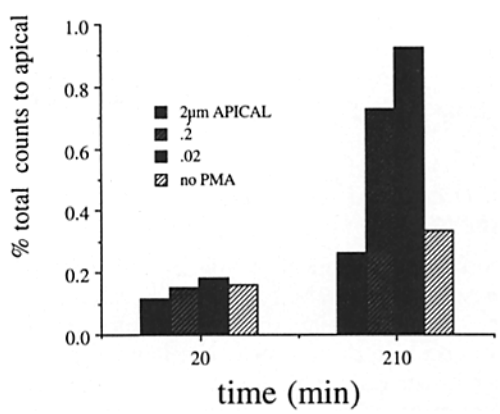

Figure 5. Effect of PMA on paracellular leakage across MDCK cell monolayers. MDCK cells were grown on filters for 4 d. 2 μCi of [^{14}C]inulin was added to the basal medium at the same time that PMA at final concentrations of 2.0, 0.2, 0.002 μM, or no PMA was added to both the apical and basal media. Apical medium was collected at 20 and 210 min and counted. Basal medium was counted at 210 min. Apical [^{14}C]inulin is represented as a percent of total radioactive material.

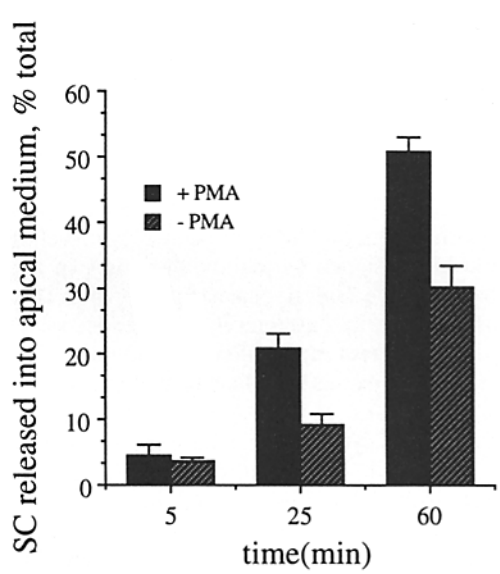

Figure 6. Pulse–chase analysis of the effect of PMA on apical release of SC. Cells were cultured on filters, metabolically labeled with [^{35}S]Cys for 15 min at 37°C, and chased for 1 h in MEM. Cells were then chased in MEM with or without 2 μM PMA. Apical medium was collected at 5, 25, and 60 min. Basal medium was collected only at 60 min. Cells were lysed. All samples were subject to immunoprecipitation with antibody to the SC portion of the pIgR and the immunoprecipitates processed for SDS-PAGE. Gels were analyzed with a Molecular Dynamics Phosphorimager. SC delivered apically is represented as a percentage of the total initially synthesized pIgR. Values are the mean ± SEM using four filters from one experiment. Similar results were obtained in three separate experiments.

of the pIgR without ligand bound. Previous work has shown that after synthesis the pIgR is delivered within 60 min to the basolateral surface and then transcytosed to the apical surface where it is either cleaved into SC or internalized (Breitfeld et al., 1989). We therefore metabolically labeled the pIgR in a 15-min pulse with [^{35}S]Cys and then chased for 60 min to allow the pIgR to reach the basolateral surface. Some pIgR molecules will have been endocytosed and entered the transcytotic pathway during this period. We then added 2 μM PMA and continued the chase for a second period of up to 60 min. As shown in Fig. 6, release of SC is stimulated by the PMA treatment. This indicates that transcytosis of the empty pIgR is also stimulated by PMA.

Phosphorylation of pIgR at Ser664 or Ser726 Is Not Required for PMA Stimulation

We investigated whether phosphorylation of Ser^{664} or Ser^{726} is necessary for PMA-mediated stimulation of transcytosis by examining the effect of PMA on transcytosis of a mutant pIgR in which both serines have been converted to alanines (pIgR Ala^{664,726}). The construction and characterization of this mutant pIgR will be described elsewhere (Okamoto, C., W. Song, and K. Mostov, unpublished results). Briefly, the mutant pIgR is internalized about three times more slowly than the wild type pIgR. However, the mutant appears to behave nearly identically to the pIgR WT in all other measured trafficking parameters, including delivery from the TGN to the basolateral surface and transcytosis of pre-inter-

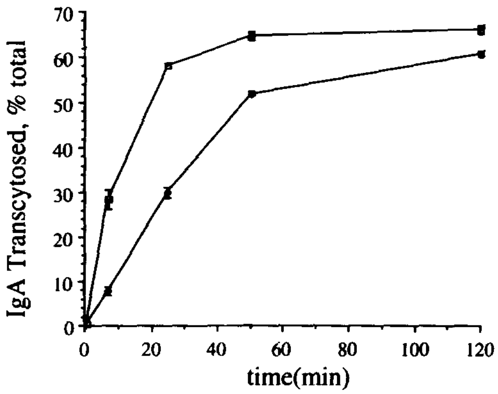

Figure 7. Effect of PMA on transcytosis of IgA by cells expressing the S664A-S726A mutant pIgR. MDCK cells expressing this mutant pIgR were allowed to internalize ^{125}I-labeled dIgA from the basal surface for 10 min at 37°C and then washed and chased for 120 min in the presence or absence of 2 μM PMA. Apical and basal media were collected and counted. Values are means ± SEM for six filters from two separate experiments. Similar results were obtained in four separate experiments.

nalized ^{125}I-labeled dIgA. We found that the rate of transcytosis of pre-internalized ^{125}I-labeled dIgA by the mutant was increased by PMA (Fig. 7). This indicates that phosphorylation of the two mutated serine residues is not required for the stimulation of transcytosis.

PMA May Stimulate Transcytosis by Activation of PKC α and/or ϵ Isozymes

Treatment of cells with PMA directly activates PKC by mimicking DAG. While DAG is quickly degraded and only transiently present on membranes, PMA is not rapidly degraded and therefore able to persistently activate PKC. The effect of prolonged exposure to PMA is degradation of the membrane-bound (activated) PKC molecule (Young et al., 1987). This secondary effect of PMA allows it to be used to down regulate certain PKC isozymes. We used this approach to identify the PKC isozymes involved in PMA-mediated stimulation of transcytosis. Fig. 8 shows that after a 16 h pre-treatment of cells with 2 μM PMA transcytosis was no longer stimulated by PMA. This treatment did not however, affect the basal rate of transcytosis. Pretreated cells were also able to endocytose dIgA at the same rate as non-treated cells.

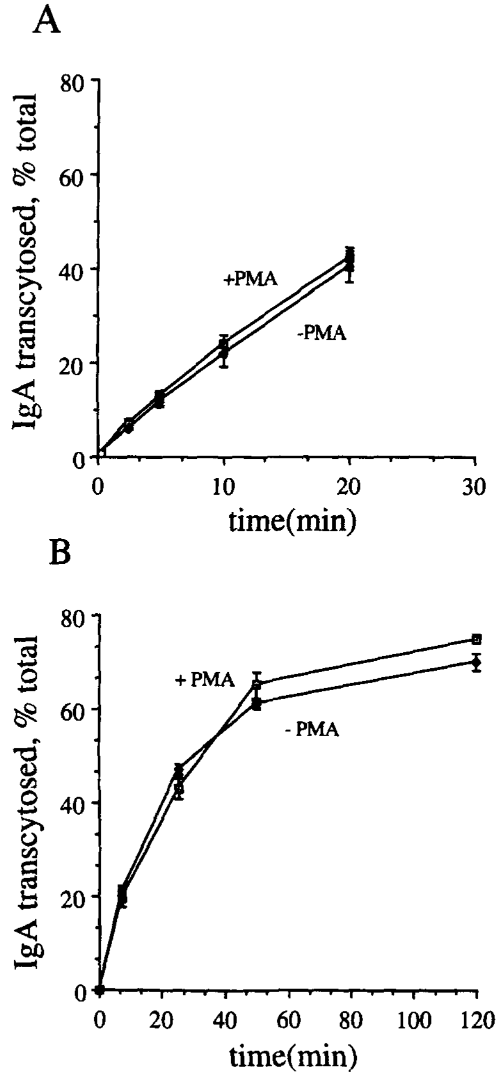

Figure 8. Effect of prolonged treatment with PMA on transcytosis of dIgA. Cells were treated with 2 μM PMA for 16 h prior to the dIgA transcytosis assay. ^{125}I-labeled dIgA ligand was internalized for 10 min at 37°C. Cells were washed and chased in MEM at 37°C with or without 2 μM PMA for up to 2 h (Fig. 6 *A*, early time points up to 20 min; Fig. 6 *B*, time points up to 2 h). Apical and basal media were collected and counted as above. Values are mean ± the SEM, $N = 5$.

We analyzed the MDCK cells by Western blot analysis to determine which PKC isozymes were present and which were stimulated or down regulated by PMA treatment. Five of the available isozyme specific anti-PKC antibodies reacted with proteins from MDCK cells (data not shown), suggesting the presence of the α, β, δ, ϵ, and ζ isozymes. We also tested for the presence of the brain specific γ isozyme and as expected did not detect it in MDCK cells. To measure the translocation and down regulation of PKC isozymes by PMA, Western blot analysis of cytosol and particulate fractions were performed. Translocation of an isozyme from the cytosol to a particulate fraction indicates activation (Kraft and Anderson, 1983). Down regulation of an isozyme is evident by a reduction of isozyme present in either cell fraction after prolonged PMA treatment. We determined which of these isozymes was translocated to the particulate fraction following a 5-min treatment with PMA. As shown in Fig. 9, the α and ϵ isozymes were translocated upon exposure to PMA. Down regulation of the α isozyme appears to begin during the 5-min exposure. This apparent rapid down regulation is not inconsistent with the rapid stimulation of transcytosis. Prolonged treatment (16 h) of the cells with PMA caused the α and to a lesser extent the ϵ isozymes to be depleted from the cells. The ζ isozyme did not translocate or down regulate in response to PMA. This non-responsiveness to phorbol ester is consistent with previous studies (Ono et al., 1989). In our hands the signals for the β and δ isozymes were not strong enough to reliably determine translocation or down regulation by PMA. Nevertheless, previous work by others has shown that in MDCK cells the β isozyme

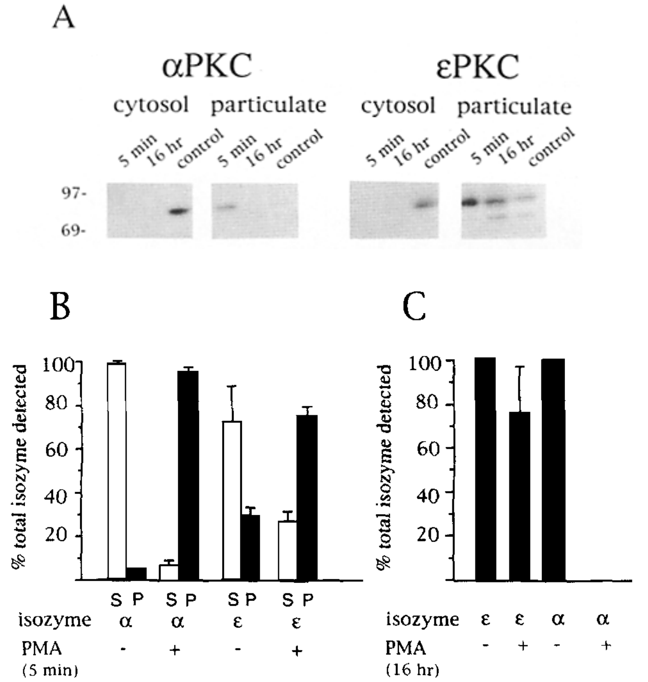

Figure 9. (*A*) MDCK cells treated with 2 μM PMA for 5 min, 16 h, or untreated were fractionated into cytosolic and particulate fractions as described in the text. Equal amounts of protein from each fraction were subjected to 10% SDS-PAGE and analyzed by Western blotting using antibodies against PKC α and ε. (*B*) Bands representing the isozymes from the particulate and cytosol fractions of cells treated with 2 μM PMA for 5 min were quantitated by phosphorimaging as described in the text and are displayed as the mean of three separate experiments ± SEM. Values are normalized so that the total in pellet and supernatant = 100%. (*C*) The ratio of total PKC isozymes α, ε from cells treated with PMA for 16 h compared to total α and ε from untreated cells. Values are the mean of three separate experiments ± SEM, normalized so that the values from untreated cells = 100%.

is neither down regulated nor translocated by phorbol ester (Godson et al., 1990). Taken together these data are consistent with the hypothesis that the increased rate of transcytosis in response to PMA may be mediated by PKC α or ε isozymes, or both. However, we can not rule out that the effect of PKC is (at least partly) via some other PKC isozymes.

Transcytosis but Not Recycling of Transferrin Is Stimulated by 2 μM PMA

We asked if PMA would affect the rate of transcytosis or recycling of another ligand internalized from the basal surface. We used transferrin (Tf) a classical basolateral recycling protein. Ordinarily >95% of basolaterally endocytosed Tf is recycled to that surface (Fuller and Simons, 1986). However, we have recently shown that at least some of the basolaterally endocytosed Tf reaches the same apical recycling compartment as transcytosing dIgA before the Tf recycles to the basolateral surface. To examine the effect of PMA on Tf recycling and transcytosis we pre-internalized ^{125}I-labeled Tf from the basolateral surface of MDCK cells for one hour at 37°C, washed at 4°C and chased with MEM containing 10 μg/ml of unlabeled Tf with or without 2 μM PMA. Fig. 10 *A* shows that the extent of transcytosis of transferrin is stimulated two fold over the 2-h time course while the half-time for transcytosis is relatively unchanged. In contrast, with dIgA PMA increases the initial rate of apical release of dIgA, but has little effect on the final extent of transcytosis. This difference may reflect the different routes that these ligands take to reach to the apical recycling compartment (see Discussion). Fig. 10 *B* shows that recycling of Tf is slightly diminished so that the material remaining associated with the cells is not altered by PMA (Fig. 10 *C*). This data indicates that PMA increases the extent of apical release of basolaterally internalized transferrin.

Discussion

We have found that PMA stimulates transcytosis of the pIgR and dIgA bound to the pIgR. We measured this stimulation in three ways: transcytosis of ^{125}I-labeled dIgA, morphologic analysis of dIgA in cells, and release of SC from pIgR. All three methods gave similar results. Stimulation appears to be very rapid, occurring within the first few minutes of adding PMA.

Previous work (Barroso and Sztul, 1994; Apodaca, 1994) has indicated that transcytosing IgA accumulates in an apical recycling compartment prior to delivery to the apical surface. This suggests that this final delivery is a rate-limiting step in transcytosis. Our analysis has now suggested that PMA causes rapid movement of dIgA from this apical recycling compartment to the apical surface. Consistent with this, the recycling of apically endocytosed dIgA to this surface is also stimulated by PMA. These results raise the possibility that PKC may regulate delivery from the apical recycling compartment to the apical surface. Analysis by confocal microscopy suggested that while the predominant effect of PMA is at the apical recycling compartment, an earlier step in transcytosis (perhaps movement of IgA from basolateral early endosomes to the apical recycling compartment) is also accelerated by PMA, particularly at longer time points. Moreover, transcytosis of basolaterally internalized Tf is stimulated by PMA, suggesting that the effect is not limited to IgA. This may be due to stimulation of transcytosis of Tf that has reached the apical recycling compartment and/or increased delivery of Tf from basolateral early endosomes to this compartment. Notably, recycling from basolateral endosomes to the basolateral surface is not stimulated by PMA, indicating that the basolateral and apical endosome systems differ in at least one key respect.

How general is the phenomenon that we have described? Transcytosis of IgA in rat hepatocytes also appears to involve a tubular compartment underneath the apical (bile canalicular) plasma membrane (Geuze et al., 1984; Hoppe et al., 1985). Cell fractionation experiments have suggested that this compartment contains material endocytosed from the apical surface (Quintart et al., 1989), so it is likely that this compartment is analogous to the apical recycling compartment that we have characterized in MDCK cells. Regulation of transcytosis by rat hepatocytes has not, to our knowledge, been investigated.

However, many other types of epithelial cells appear to contain a similar specialized apical recycling compartment. Regulation of delivery from this compartment to the surface is crucial for cellular function (Coleman and Wade, 1992;

Kelly, 1993*a*). Plasma membrane transporter molecules (e.g., for water and ions) can be taken up from the apical surface, stored in this compartment, and reinserted into the apical plasma membrane in response to stimulation by hormones or neurotransmitters. Similar compartments have been suggested to exist in other cell types, including adipocytes, neurons, and fibroblasts (Kelly, 1993*a*). In particular, in neurons a homologue of the apical recycling compartment may exist near axon terminals. Our data suggest that the apical recycling compartment that contains transcytosing dIgA, and perhaps some Tf, may be analogous to this hormone or neurotransmitter regulated compartment. The regulation of delivery from the apical recycling compartment to the apical surface by PKC in MDCK cells may therefore be an example of a widespread phenomenon.

Moreover, in a variety of non-polarized cell lines PMA has been shown to affect the movement of numerous other receptors (Backer and King, 1991). One of the best studied examples is the Tf receptor (TfR). In mouse 3T3, CHO, and Hep G2 cells PMA causes an increase in TfR on the cell surface, perhaps due to an increased rate of externalization (Rothenburger et al., 1987; Zerial et al., 1987; Fallon et al., 1988; McGraw et al., 1988). This PMA-induced externalization of TfR may be similar to the PMA-stimulated transcytosis of Tf that we have observed. Although PMA can induce phosphorylation of the TfR by PKC, mutation of the phosphorylated Ser at position 24 of the cytoplasmic tail does not prevent the externalization of the TfR (McGraw et al., 1988). This seems to be analogous to our results with the pIgR.

Stimulation of transcytosis of dIgA by hormones and neurotransmitters apparently occurs in vivo, suggesting that our results are not simply an artifact of using PMA and cultured cells. Bombesin, cholecystokinin, and pilocarpine (a cholinergic agonist) stimulate IgA secretion into rat intestine (Jin et al., 1989; Wilson et al., 1992; Freier et al., 1987), while methacholine stimulates IgA transport into human nasal secretions (Raphael et al., 1988). Stimulation of dIgA transcytosis in response to hormones may be a way to regulate delivery of dIgA to mucosal surfaces in response to the changing needs of the organism. In very recent preliminary experiments we have found that transcytosis of ^{125}I-labeled dIgA by MDCK cells expressing the pIgR can be stimulated by treatment with bradykinin, carbachol, or epinephrine (unpublished data). The stimulation by these agents is very similar to that observed with PMA. Stimulation of transcytosis in vivo by cholinergic agonists or in MDCK cells by bradykinin, carbachol or epinephrine may involve PKC activation, though this has not been demonstrated. Bradykinin, carbachol, and epinephrine do activate inositol 1,4,5-triphosphate and DAG production in MDCK cells (Weiss et al., 1989).

Our experimental system allowed us to show that PMA-mediated stimulation of transcytosis does not require phosphorylation of Ser^{664} or Ser^{726}, the two main sites of phosphorylation of the pIgR. Transcytosis of a mutant pIgR where both residues were converted to alanine was still stimulated. If cells expressing this double mutant pIgR are incubated with [^{32}P]ortho-phosphate, the incorporation of ^{32}P into the mutant pIgR is reduced by >90%, relative to the wild-type pIgR (Okamoto, C., W. Song, M. Cardone, and K. Mostov, unpublished). There is still some incorporation of ^{32}P by the mutant pIgR, indicating that there is at least one undiscovered minor site of phosphorylation. However, ^{32}P labeling of the mutant receptor was not significantly altered by PMA treatment (data not shown) further

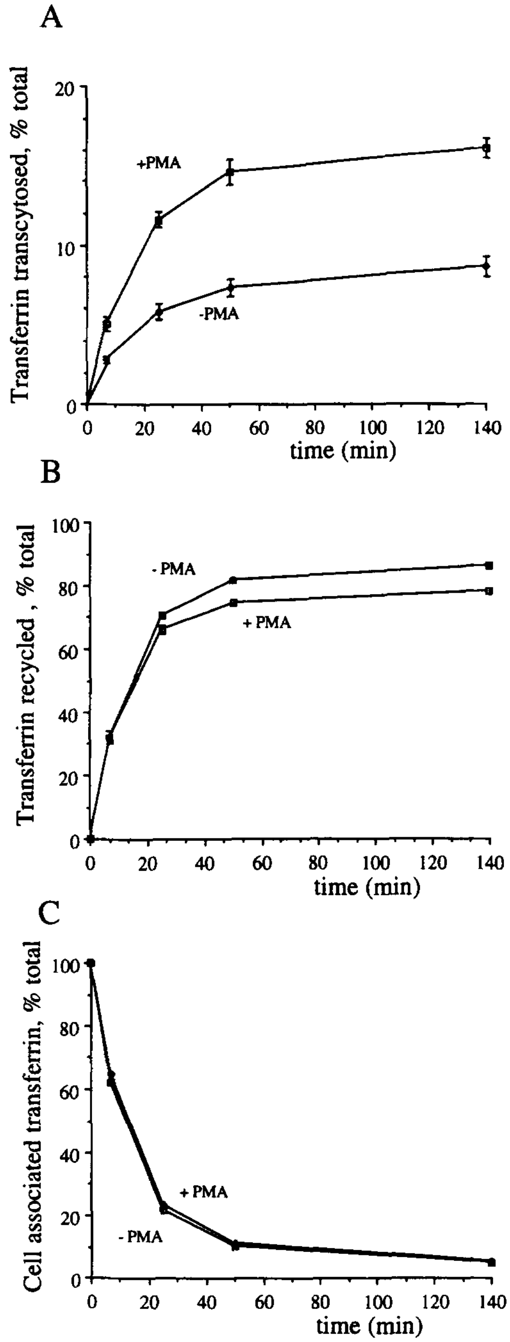

Figure 10. Effect of PMA on transcytosis and recycling of transferrin. MDK cells were allowed to internalize ^{125}I-labeled canine transferrin from the basal surface for 1 h at 37°C, washed at 4°C and then chased in MEM containing 10 μg/ml unlabeled ligand for up to 140 min. Samples were taken from the apical and basal media at intervals indicated and counted. *A* indicates the transferrin that was released into the apical medium, expressed as a percent of initially internalized ligand. Similarly, *B* represents the transferrin that was recycled to the basolateral medium, while *C* represents the internalized transferrin that remained associated with the cells at each time point. Values are means ± SEM for four filters. Similar results were obtained in three separate experiments.

supporting the notion that the pIgR is not a substrate for either PKC or another kinase that may be activated by PKC.

The stimulation of transcytosis by PMA is probably due to activation of PKC by PMA. Prolonged exposure to PMA prevented the effect of PMA on transcytosis. This is characteristic of effects of PMA that are mediated by PKC. However, we cannot exclude other possible targets for PMA (Ahmed et al., 1993). The effect of PMA may be due to activation of PKC isozymes α and/or ϵ. Of the various PKC isozymes that we could reliably detect in MDCK cells, only these two were translocated by PMA from the cytosolic to the particulate fraction and were depleted from the cells after long PMA exposure. These results suggest that either of these two isozymes may be involved in the PMA-mediated stimulation of transcytosis.

A model explaining how PKC activation by PMA could lead to stimulation of transcytosis is suggested by the recent report that activation of PKC by PMA promotes the binding of ADP-ribosylation factor (ARF) and coatomer to Golgi membranes (De Matteis et al., 1993). Although the exact roles of ARF and coatomer are under investigation, these are components of non-clathrin–coated vesicles involved in protein trafficking through the Golgi, and perhaps elsewhere in the cell. The binding of coatomer and ARF to Golgi membranes is prevented by brefeldin A (BFA). PMA antagonized this effect of BFA, while down regulation of PKC by prolonged treatment with PMA enhanced this effect of BFA. Moreover, PMA enhanced constitutive secretion from the TGN to the cell surface. We do not know much about the molecular mechanism of transcytosis. However, BFA blocks transcytosis of dIgA by the pIgR, suggesting that ARF and coatomer, or related molecules are involved (Hunziker et al., 1991). One possible model is that by analogy with its effects on the Golgi, PMA-activated PKC may enhance binding of ARF and a coatomer (or related proteins) to a compartment involved in transcytosis, and thereby stimulate transcytosis.

A major difference between our results and those of De Matteis et al. (1993) is that they concluded that the PKC β isozyme was responsible for the effects observed in their system, whereas in our system, the PKC α and/or ϵ isozymes were probably involved. The roles of various isozymes of PKC are just beginning to be explored. Our results and those of De Matteis et al. (1993) suggest that different PKC isozymes may regulate different steps in membrane traffic.

The actin cytoskeleton is also regulated by PKC (Hartwig et al., 1992; Hutari et al., 1992; Luna and Hitt, 1992). Therefore, another possible, non-mutually exclusive mechanism for the effect of PMA on transcytosis is that PMA induces cytoskeletal rearrangements that promote vesicle fusion with the apical plasma membrane (reviewed in Kelly, 1993*b*). This model would be analogous to the mechanism proposed for PMA stimulation of exocytosis from regulated secretory granules. Several preliminary observations that we have made argue against this model, however. Phalloidin rhodamine staining of actin showed that while the stress fibers in the basal region of the treated MDCK cells were affected by treatment of the cells with 2 μM PMA, the apical cortical actin seemed to be unaffected. In addition, cytochalasin treatment of cells did not affect the rate or extent of transcytosis of dIgA (data not shown). If PMA-induced rearrangement of the apical actin meshwork was responsible for the increased transcytosis this treatment should have had an effect on the apical release of IgA.

In summary we have found that PMA stimulates delivery to the apical surface of membrane-bound molecules endocytosed from both the basolateral (pIgR, Tf) and apical (pIgR) surfaces of MDCK cells. PMA appears to stimulate apical delivery from a recently described apical recycling compartment, which is accessible to ligands endocytosed from both surfaces. The effect of PMA on transcytosis is thus domain specific and may indicate a stimulation by PMA may be via activation of PKC α and/or ϵ isozymes.

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